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Phytochrome Regulates Gibberellin Biosynthesis during Germination of Photoblastic Lettuce Seeds[1]

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Germination of lettuce (*Lactuca sativa* L.) seed is regulated by phytochrome. The requirement for red light is circumvented by the application of gibberellin (GA). We have previously shown that the endogenous content of GA_1, the main bioactive GA in lettuce seeds, increases after red-light treatment. To clarify which step of GA_1 synthesis is regulated by phytochrome, cDNAs encoding GA 20-oxidases (Ls20ox1 and Ls20ox2, for *L. sativa* GA *20-ox*idase) and 3β-hydroxylases (Ls3h1 and Ls3h2 for *L. sativa* GA *3β-h*ydroxylase) were isolated from lettuce seeds by reverse-transcription polymerase chain reaction. Functional analysis of recombinant proteins expressed in *Escherichia coli* confirmed that the Ls20ox and Ls3h encode GA 20-oxidases and 3β-hydroxylases, respectively. Northern-blot analysis showed that Ls3h1 expression was dramatically induced by red-light treatment within 2 h, and that this effect was canceled by a subsequent far-red-light treatment. Ls3h2 mRNA was not detected in seeds that had been allowed to imbibe under any light conditions. Expression of the two Ls20ox genes was induced by initial imbibition alone in the dark. The level of Ls20ox2 mRNA decreased after the red-light treatment, whereas that of Ls20ox1 was unaffected by light. These results suggest that red light promotes GA_1 synthesis in lettuce seeds by inducing Ls3h1 expression via phytochrome action.

Germination of lettuce (*Lactuca sativa* L. cv Grand Rapids) seed is regulated by light (Borthwick et al., 1952), a phenomenon that was paramount in the discovery of phytochrome (Butler et al., 1959). Red light induces lettuce seed germination, and far-red light given immediately after red light suppresses this effect. Phytochrome has two conformations; the first, Pr, is converted by red light to the second form, Pfr. This process is reversible by far-red irradiation (Kendrick and Kronenberg, 1994). The Pfr form is thought to be the bioactive form in the induction of lettuce seed germination. It has been demonstrated that phytochrome is encoded by a small multigene family, and it was suggested that lettuce seed germination may be regulated mainly by phytochrome B (Kendrick and Kronenberg, 1994; Shinomura, 1997).

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The GAs, a class of phytohormones that regulate various aspects of plant development, have been implicated in the induction of lettuce seed germination by light. It was shown that the requirement for red light was circumvented by the application of more than 10^{-4} M GA_3 using the intact seeds (Kahn and Goss, 1957; Ikuma and Thimann, 1960; De Greef and Fredericq, 1983). Treatment with 10^{-7} M GA_3 induced germination in the dark when the punctured seeds were used (Inoue, 1991). This difference in minimum GA_3 concentration for the induction of a saturation level of germination is probably attributable to the low permeability of GA in the structures that surround the embryo. We have previously shown that GA_1 (1,2-dihydro-GA_3) (Fig. 1) is an endogenous bioactive GA in lettuce seed: GA_1 was identified by full-scan GC-MS analysis, and treatment with 10^{-6} M GA_1 induced germination in the dark (Toyomasu et al., 1993). The endogenous content of GA_1 increased after red-light treatment, and this effect was canceled by subsequent far-red-light treatment (Toyomasu et al., 1993). Here we have focused on the mechanism by which GA_1 levels increase as a result of red-light treatment.

There are two pieces of evidence suggesting which step of GA biosynthesis is regulated by phytochrome. The germination-inducing activity of GA_{20} (Fig. 1), the immediate precursor of GA_1, is less than one-thousandth that of GA_1 in the dark (Toyomasu et al., 1993). Furthermore, endogenous levels of GA_{20} and its direct precursor, GA_{19} (Fig. 1), are much higher than that of GA_1 and are not greatly affected by light treatment (Toyomasu et al., 1993). These results suggest that conversion of GA_{20} to GA_1 is a likely key step that is regulated by phytochrome in GA biosynthesis. To examine whether the expression of genes encoding GA-biosynthetic enzymes is regulated by phytochrome, we cloned cDNAs encoding two enzymes in later steps of GA_1 biosynthesis.

GAs are diterpenoid compounds produced from geranylgeranyl diphosphate through a complex biosynthetic pathway. Recently, cDNAs encoding several of the GA-biosynthetic enzymes have been isolated and characterized: copalyl diphosphate synthase (Sun and Kamiya, 1994), *ent*-kaurene synthase (Yamaguchi et al., 1996), GA 7-oxidase (Lange, 1997), GA 20-oxidase (Lange et al., 1994), and GA 3β-hydroxylase (Chiang et al., 1995). GA

Abbreviation: RACE, rapid amplification of cDNA ends.

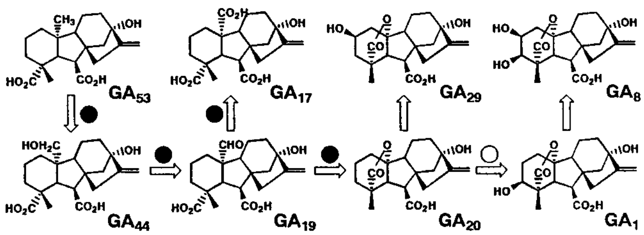

Figure 1. Early 13-hydroxylation GA-biosynthetic pathway in higher plants. ●, Steps catalyzed by GA 20-oxidase; ○, steps catalyzed by 3β-hydroxylase. GA_1, GA_{17}, GA_{19}, and GA_{20} have been identified in extracts of lettuce seeds (Toyomasu et al., 1993).

20-oxidase and GA 3β-hydroxylase are soluble, 2-oxoglutarate-dependent dioxygenases that catalyze the conversion of $GA_{53} \rightarrow GA_{44} \rightarrow GA_{19} \rightarrow GA_{20}$ and $GA_{20} \rightarrow GA_1$, respectively. This early 13-hydroxylation pathway (Fig. 1) has been shown to be present in lettuce seeds (Toyomasu et al., 1993).

We report here the cloning of cDNAs encoding GA 20-oxidases and 3β-hydroxylases from lettuce seeds and investigate the effects of light on their expression levels.

MATERIALS AND METHODS

Light Sources and Plant Materials

Red light (5 W m^{-2}) and far-red light (4.5 W m^{-2}) were as described previously (Yang et al., 1995). Lettuce (*Lactuca sativa* L. cv Grand Rapids) seeds were obtained from South Pacific Seeds (New South Wales, Australia) in 1996 and stored at 20°C with silica gel in the desiccator until they were used. Seeds (0.5 g) were incubated in the dark at 25°C for 3 h in a Petri dish (6 cm i.d.) containing 2 mL of buffer (0.1 mM Mes, pH 6.1), and then the buffer was removed and 1.5 mL of fresh buffer was added. Three types of light treatments were given: (a) far-red light, (b) far-red light followed by red light, and (c) far-red light, red light, and far-red light, successively. Each irradiation was carried out for 15 min. After each light treatment the seeds were incubated in the dark at 25°C. The seeds were harvested at hourly intervals up to 8 h after each light treatment and frozen in liquid nitrogen. Seeds incubated in the dark for 3 h were also harvested (0 h). All of these procedures were carried out under dim-green light.

Reverse-Transcription PCR

Two degenerate primers for GA 20-oxidase described previously (Toyomasu et al., 1997) were used: 5′-AAI(TC)TICCITGGAA(AG)GA(AG)AC-3′ (sense primer) and 5′-TTIGG(AG)CAIA(AG)(AG)AA(AG)AAIGC-3′ (antisense primer). The design of degenerate primers for GA 3β-hydroxylase was based on conserved amino acid regions of GA 3β-hydroxylase of Arabidopsis (Chiang et al., 1995), pumpkin (Lange et al., 1997), and pea (Lester et al., 1997; Martin et al., 1997). The sequences of these oligonucleotides were 5′-ATGTGGT(AC)IGA(AG)GGITT(CT)-AC-3′ (sense primer, encoding MW[SY]EGFT) and 5′-(GT)GIGGICCIIAIA(AG)(AG)(AT)AIGC-3′ (antisense primer, encoding A[FY][FL][FWY]GP[PQ]). Total RNA was extracted from the frozen lettuce seeds at 0 h and 1 to 3 h after far-red-/red-light treatments, and double-stranded cDNA was synthesized according to the methods described previously (Toyomasu et al., 1997). Twenty nanograms of each double-stranded cDNA was used as a template for PCR. The reaction mixture (100 μL) contained 200 μM deoxyribonucleotide triphosphate, 1.5 mM $MgCl_2$, 1 μM of each primer, and 2.5 units of Expand HF (Boehringer Mannheim). Samples were heated to 95°C for 2 min, then subjected to 40 cycles of 94°C for 1 min, 45°C for 1 min, and 72°C for 1 min, with final extension for 7 min.

PCR of 5′ Ends of cDNAs

5′-RACE was carried out using a cDNA amplification kit (Marathon, Clontech, Palo Alto, CA). The first-strand cDNA was synthesized from poly(A^+) RNA using each gene-specific primer (antisense). Double-stranded cDNA with an adaptor was prepared according to the supplier's instructions and subjected to PCR using the adaptor primer (5′-CCATCCTAATACGACTCACTATAGGGC-3′; AP1, Clontech) enclosed in the cDNA amplification kit and another gene-specific primer (antisense). The PCR conditions were the same as those described above except that the annealing temperature was 64°C. The design of the gene-specific primers (not shown) was based on the nucleotide sequence of each PCR fragment.

PCR of 3′ Ends of cDNAs

First-strand cDNA was synthesized from poly(A^+) RNA using a dT primer incorporating the sequence of the adaptor primer at the 5′ end. After a 20-min treatment with RNase H, cDNA was subjected to the PCR using the adaptor primer and the gene-specific primer (sense).

PCR of Full-Length cDNAs

Double-stranded cDNAs described in the section on reverse-transcription PCR were also used as templates. PCR was carried out using the 5′ and 3′ end primers to amplify the coding region. Each primer consisted of a gene-specific sequence and incorporated a restriction enzyme site at its 5′ end. The PCR conditions were as described above except that the annealing temperature was 50°C and the extension time was 1.2 min.

Cloning and Sequence Analysis of PCR Products

PCR products were purified by agarose-gel electrophoresis and ligated into a pCRII vector using the TA cloning kit (Invitrogen, San Diego, CA). The ligation products were introduced into *Escherichia coli* JM109, and recombinant clones were selected. The nucleotide sequence of each clone was determined using a *Taq*-cycle sequencing kit (Dye Primer, Applied Biosystems) and a DNA sequencer (model ABI 377, Applied Biosystems). For each full-length cDNA for expression analysis, the insert was excised from the plasmid using the appropriate restriction enzymes and inserted into the pMAL-c2 vector (New England Biolabs).

Sequence Similarity Search and Alignment of Amino Acid Sequences

Homology searches of the databases were performed using the Basic Local Alignment Search Tool of the National Center for Biotechnology Information (http//www.ncbi.nlm.nih.gov/BLAST/). Alignments of amino acid sequences were carried out using the Clustal W program (http//www.clustalw.genome.ad.jp/).

Heterologous Expression in *E. coli* and Enzyme Assay

Recombinant clones for expression analysis were grown by shaking at 37°C in 150 mL of 2×YT medium (1.6% Bacto Tripton, 1% yeast extract, and 0.5% NaCl) with ampicillin (100 μg/mL) to the midlogarithmic phase. Expression was induced by the addition of isopropyl-D-thiogalactopyranoside to 1 mM, and cultures were grown for an additional 22 h at 18°C and harvested. The cell pellets were resuspended (0.2 g/mL) in 50 mM Tris-HCl, pH 8.0, buffer containing 5 mM DTT and 10% (v/v) glycerol, and then frozen in liquid nitrogen. After thawing on ice, the cell suspension was treated with lysozyme (0.1 mg/mL) and disrupted by sonication. The lysate was centrifuged, and the resulting supernatant was collected and used for enzyme assays. The enzyme preparations were assayed for enzyme activity by incubation with GAs (200 ng) at 30°C for 1 h under the conditions described previously (Toyomasu et al., 1997). GA_{53} and GA_{20} were purchased from Prof. L.N. Mander (Australian National University, Canberra) and used as substrates. After methyl ester-trimethylsilyl ether derivatization, products were subjected to full-scan analysis using a gas chromatograph-mass spectrometer (Finnigan MAT, San Jose, CA). GC-MS conditions were as described previously (Kawaide et al., 1995).

Southern- and Northern-Blot Analyses

Genomic DNA, digested with restriction enzymes and separated on a 1% (w/v) agarose gel, was transferred onto a nylon membrane (Hybond N^+, Amersham) using standard blotting techniques (Sambrook et al., 1989). Membranes were prehybridized for 3 h at 68°C and hybridized with a ^{32}P-labeled PCR fragment for 18 h at 68°C in a rapid hybridization buffer (Amersham). The membrane was washed successively at 68°C with 2× SSC/0.1% (w/v) SDS for 10 min, 1× SSC/0.1% SDS for 1 h, and 0.2× SSC/0.1% SDS for 1 h. Radioactivity was recorded on an imaging plate using an analyzer (BAS-2000, Fujix, Tokyo, Japan). Total RNA was extracted from frozen lettuce seeds by the SDS-phenol method described by Sambrook et al. (1989). Total RNA (50 μg/lane) was denatured and electrophoresed in a 1% (w/v) agarose/2.2 M formaldehyde gel. Blotting, hybridization, washing, and exposure were carried out as described above. The northern-blot analysis was repeated with at least two independent preparations of RNA.

RESULTS

Reverse-Transcription PCR with Degenerate Primers for GA 20-Oxidase and 3β-Hydroxylase

Degenerate primers for GA 20-oxidase and 3β-hydroxylase were designed on the basis of published sequences from other plant species, as described in "Materials and Methods." To clone all genes encoding these enzymes that may be expressed during germination, we prepared samples from seeds just before the light treatment (0 h), and 1, 2, and 3 h after the far-red-/red-light treatment. PCR with degenerate primers was carried out using cDNA derived from poly(A^+) RNA for each sample. The bands of the expected size, approximately 530 bp, were amplified by PCR using the GA 20-oxidase primers. Sequence analysis of the PCR products indicated that two different fragments of 531 bp were obtained, which were derived from different GA 20-oxidase genes and named Ls20ox1 and Ls20ox2 (<u>L</u>. <u>s</u>ativa GA <u>20-ox</u>idase). The PCR using the GA 3β-hydroxylase primers amplified products of the predicted size, approximately 520 bp. The DNA sequence of 506- and 515-bp products indicated that they were derived from different GA 3β-hydroxylase genes and named Ls3h1 and Ls3h2 (*<u>L</u>. <u>s</u>ativa* GA <u>3β-h</u>ydroxylase), respectively.

Isolation of Full-Length cDNAs

RACE was performed to determine the full-length cDNA sequence using gene-specific primers based on the nucleic acid sequence of each PCR fragment. Each open reading frame was amplified using primers based on the tentative nucleic acid sequence to check the fidelity of the sequence determined by RACE and to express in *E. coli*. The predicted coding regions of Ls20ox1, Ls20ox2, Ls3h1, and Ls3h2 were 1146, 1107, 1089, and 1086 bp, respectively, encoding products of 383, 369, 363, and 362 amino acids, respectively. Homology searches indicated that the derived amino acid sequences of Ls20ox1, Ls20ox2, Ls3h1, and Ls3h2 have high levels of similarity to other plant GA 20-oxidases and 3β-hydroxylases, respectively (Fig. 2). For example, identities of Ls20ox1/Ps074, Ls20ox2/Ps074, Ls3h1/Ps3h, and Ls3h2/Ps3h are 70%, 69%, 63%, and 60%, respectively. Homologous clones from lettuce showed greater similarity to each other, with identities of Ls20ox1/Ls20ox2 and Ls3h1/Ls3h2 of 80% and 70%, respectively.

Functional Analysis of GA 20-Oxidases and 3β-Hydroxylases from Lettuce Seeds

Each full-length cDNA was expressed in *E. coli* to yield recombinant protein in a fusion with maltose-binding protein. Because only 13-hydroxy-GAs were identified from the lettuce seeds (Toyomasu et al., 1993), only the 13-hydroxy-GAs, GA_{53} and GA_{20}, were used as substrates in enzyme assays using recombinant proteins. Both recombi-

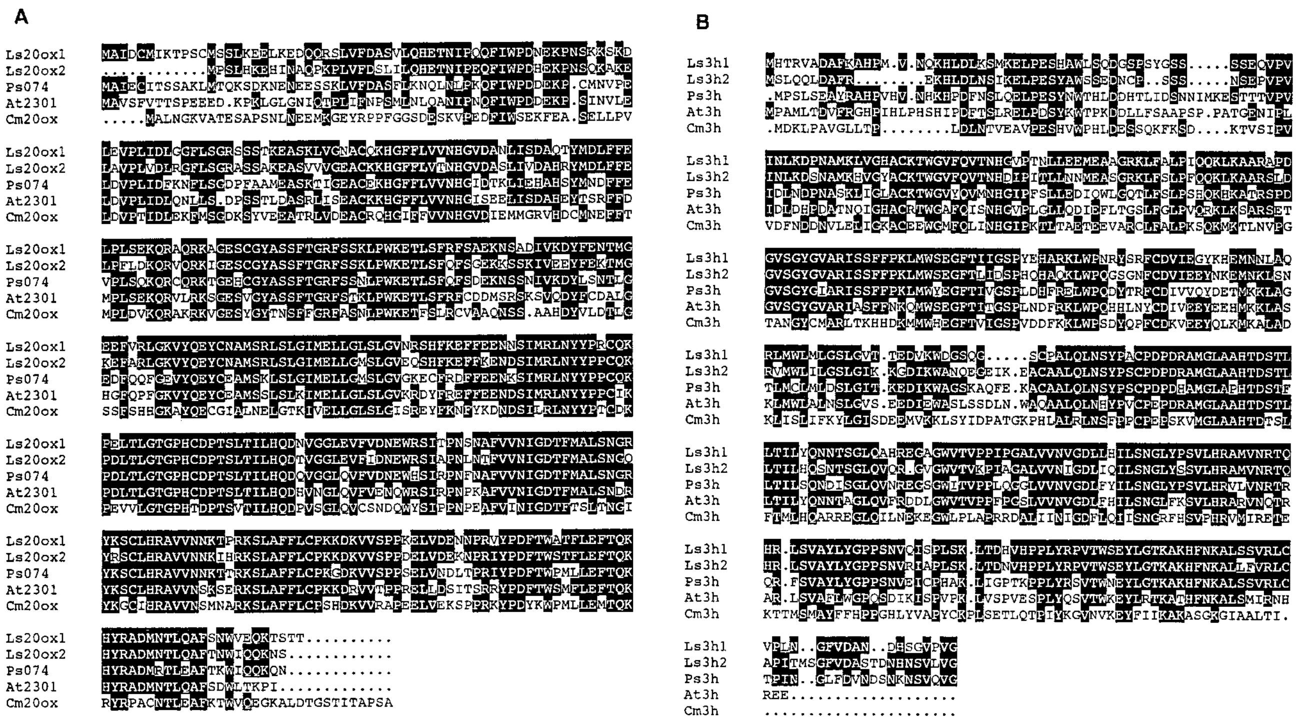

Figure 2. Alignment of deduced amino acid sequences of GA 20-oxidases (A) and 3β-hydroxylases (B) from lettuce seeds with GA-biosynthetic enzymes from other plants (Ps, pea; At, Arabidopsis; and Cm, pumpkin). Dark shading indicates identical residues. The sequence data of Ls20ox1, Ls20ox2, Ls3h1, and Ls3h2 will appear in the databases with the accession numbers AB012203, AB012204, AB012205, and AB012206, respectively. Ps074 (no. X91658) (Martin et al., 1996), At2301 (no. X83379) (Phillips et al., 1995), and Cm20ox (no. X73314) (Lange et al., 1994) are GA 20-oxidases, and Ps3h (Le; no. AF001219) (Martin et al., 1997), At3h (GA4; no. L37126) (Chiang et al., 1995), and Cm3h (no. U63650) (Lange et al., 1997) are GA 3β-hydroxylases from pea, Arabidopsis, and pumpkin, respectively.

nant proteins of Ls20ox1 and Ls20ox2 converted GA_{53} to GA_{20}, with no GA_{17} (tricarboxylic acid type; Fig. 1) being formed (Table I). The recombinant proteins from Ls3h1 and Ls3h2 hydroxylated GA_{20} at C-3 to produce GA_1 (Table I). GA_{29} and GA_8 (Fig. 1) were not detected in the products, indicating that the recombinant proteins did not possess any 2β-hydroxylase activity against GA_{20} or its product, GA_1 (Table I).

Southern-Blot Analysis

The four clones were characterized further by Southern-blot analysis using the corresponding PCR fragments as probes. The single-band pattern produced by restriction of genomic DNA from lettuce (Fig. 3) showed that there was no cross-hybridization between the clones nor hybridization with any other related sequences in the lettuce genome.

Northern-Blot Analysis

The gene expression pattern of the four GA-biosynthetic enzymes was characterized in the lettuce seeds up to 8 h after light treatment (Fig. 4). Three kinds of light treatments were carried out, as described in "Materials and Methods," with details as described previously (Toyomasu et al., 1993). The far-red-light treatment was used as the control because far-red light suppresses the low level of germina-

Table I. *GC-MS data of the methyl ester-trimethylsilyl ether derivatives of products after incubation of extracts of E. coli expressing fusion proteins derived from Ls20ox and Ls3h with GA_{53} and GA_{20}, respectively*

Clone	Substrate	Identified Metabolite	Retention Time on GC	Principal Ions/Relative Abundance
			min:s	*% of base peak*
Ls20ox1	GA_{53}	GA_{20}	7:18	418 (M^+, 100), 403 (13), 375 (63), 359 (25), 301 (21), 235 (6), 207 (30)
Ls20ox2	GA_{53}	GA_{20}	7:18	418 (M^+, 100), 403 (15), 375 (70), 359 (23), 301 (22), 235 (7), 207 (29)
		Authentic GA_{20}	7:18	418 (M^+, 100), 403 (11), 375 (30), 359 (16), 301 (6), 235 (6), 207 (22)
Ls3h1	GA_{20}	GA_1	8:10	506 (M^+, 100), 491 (7), 448 (17), 377 (10), 376 (11), 313 (10), 207 (13)
Ls3h2	GA_{20}	GA_1	8:09	506 (M^+, 100), 491 (8), 448 (20), 377 (8), 376 (17), 313 (11), 207 (10)
		Authentic GA_1	8:09	506 (M^+, 100), 491 (9), 448 (26), 377 (10), 376 (18), 313 (8), 207 (10)

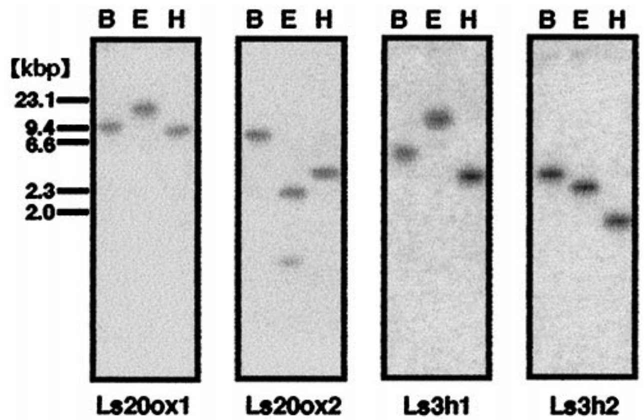

Figure 3. Southern blot of genomic DNA (10 μg/lane) from lettuce under high-stringency conditions. Temperatures for hybridization and washing were both 68°C, as described in "Materials and Methods." B, Digestion by *Bam*HI; E, digestion by *Eco*RI; and H, digestion by *Hin*dIII. Ls20ox2 probe cDNA was also digested by *Eco*RI when excised from the plasmid.

tion occurring in darkness. The far-red-/red-/far-red-light treatment was used to confirm photoreversibility of gene expression. Under conditions of far-red light and far-red/red/far-red light, Pfr is converted to Pr. Under far-red-/red-light conditions, Pr is converted to Pfr. The radicle appeared from 10 to 12 h after far-red-/red-light treatment under our experimental system.

In dry mature lettuce seeds (Fig. 4), no transcript was detected for any of the four genes. Three hours after the start of imbibition (0 h; Fig. 4), mRNAs corresponding to Ls20ox1 and Ls20ox2 were detected, and the abundance of Ls20ox1 mRNA was higher than that of Ls20ox2. Ls20ox1 mRNA levels were not markedly affected by light treatments and declined gradually during incubation. In contrast, Ls20ox2 mRNA levels decreased after far-red-/red-light treatment, and the effect was canceled by far-red light after red-light treatment (far-red-/red-/far-red-light treatment). With the 3β-hydroxylases, transcripts for Ls3h1 and Ls3h2 were not detected 3 h after the start of imbibition. Ls3h1 mRNA levels increased within 2 h after far-red-/red-light treatment, but were still not detected under conditions of Pfr removal (far-red light, far-red/red/far-red light). Ls3h2 mRNA was not detected in any samples under the highly stringent conditions of hybridization. We assume that Ls3h2 was expressed at a level below the detection limit of the northern blots, and was detected only by the more sensitive PCR process used to isolate this clone.

DISCUSSION

Light is essential for plant growth, and plants have evolved mechanisms to adapt to different light conditions, including the regulation of various aspects of growth and development by phytochrome. In some of these processes there is evidence that phytochrome acts through the GA-signaling system; however, little is known about the molecular mechanisms involved. Regulation of elongation growth by phytochrome has been investigated in a number of species. In the case of phytochrome-deficient mutants of *Brassica rapa* (*ein* mutant) (Rood et al., 1990) and sorghum (ma_3^R mutant) (Beall et al., 1991), internode elongation of pea (Campell and Bonner, 1986; Sponsel, 1986), epicotyl elongation of cowpea (Martínez-García and García-Martínez, 1992), and hypocotyl elongation of lettuce (Toyomasu et al., 1992), it was proposed that phytochrome may affect the endogenous levels of GA through its affects on GA biosynthesis and turnover. It was also suggested that phytochrome may affect the response of tissue to GA, as in epicotyl elongation of cowpea (Martínez-García and García-Martínez, 1992), mesocotyl elongation of rice (Nick and Furuya, 1993; Toyomasu et al., 1994), and hypocotyl elongation of cucumber (*lh* mutant) (López-Juez et al., 1995). Phytochrome could, therefore, affect GA biosynthesis and/or response of tissue to GA in elongation growth.

Apart from work with Arabidopsis (Hilhorst and Karssen, 1988; Yang et al., 1995) and lettuce (Inoue, 1991; Toyomasu et al., 1993), the regulation of GA action by phytochrome in seed germination has not been well studied. In Arabidopsis seeds biosynthesis of GA is necessary for germination, and phytochrome can affect the response to applied active GA (Hilhorst and Karssen, 1988; Yang et al., 1995). In the case of germination in lettuce seed, endogenous levels of GA_1 are shown to be regulated by phytochrome (Toyomasu et al., 1993). Our preliminary experiments suggest that the response of germinating lettuce seeds to active GA is not altered by phytochrome action. Inhibitors of GA biosynthesis suppress germination of the decoated seeds irradiated by red light (Inoue, 1991), and this inhibition is recovered by applied GA_1 at the same dose-response curve as that in the dark (data not shown). Therefore, regulation of lettuce seed germination by phytochrome is likely to be mediated mainly by changes in endogenous levels of bioactive GA. Thus, we investigated the relationship between GA biosynthesis and phytochrome.

Changes in the endogenous levels of GA_1, GA_{19}, and GA_{20} in lettuce seeds that had been allowed to imbibe

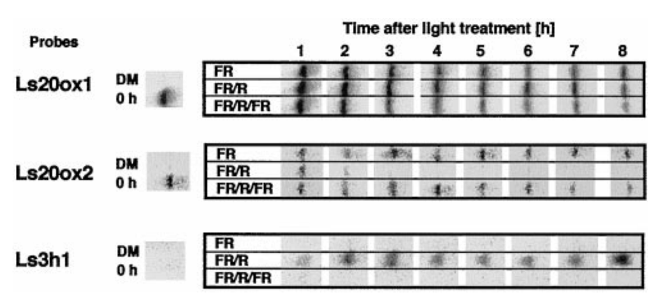

Figure 4. Gene expression of GA 20-oxidases and 3β-hydroxylases from lettuce seeds during incubation under different light conditions. Total RNA (50 μg/lane) was used for northern-blot analysis. Ls3h2 mRNA (not shown) was not detectable in any lane. DM, Mature seeds; 0 h, seeds allowed to imbibe for 3 h in the dark (just before light treatments); FR, Seeds treated with far-red light; FR/R, seeds treated with far-red/red light; FR/R/FR, seeds treated with far-red/red/far-red light. Conditions for hybridization were the same as those for Southern-blot analysis. Equal loading of RNA was checked by ethidium bromide staining of ribosomal RNAs on each gel and membrane (data not shown).

during incubation after treatment with red light and far-red light suggested that we should focus on GA 20-oxidases, particularly on 3β-hydroxylase, as possible candidates for enzymes regulated by phytochrome. We cloned cDNAs encoding these enzymes from lettuce seeds to investigate the regulation of their gene expression by phytochrome during germination. Two cDNA clones encoding GA 20-oxidases, Ls20ox1 and Ls20ox2, and two clones encoding 3β-hydroxylases, Ls3h1 and Ls3h2, were identified. It was already clear from other species that GA 20-oxidases are encoded by a small multigene family (Phillips et al., 1995; García-Martínez et al., 1997; Lange, 1997), and our results indicated that this is also true of 3β-hydroxylase. Recombinant proteins from Ls20ox1 and Ls20ox2 catalyzed consecutive steps in GA biosynthesis ($GA_{53} \rightarrow GA_{44} \rightarrow GA_{19} \rightarrow GA_{20}$) but formed no GA_{17}, despite its being detected in extracts of lettuce seeds (Toyomasu et al., 1993). It is possible that a GA 20-oxidase similar to that in immature pumpkin seeds (Lange et al., 1994) produces GA_{17} in developing lettuce seeds.

The expression patterns of the most highly expressed GA 20-oxidase and 3β-hydroxylase (Ls20ox1 and Ls3h1) in lettuce seeds corresponded well with the observed changes in endogenous levels of GA_1, GA_{19}, and GA_{20} in the seeds during incubation under different light conditions (Toyomasu et al., 1993). In particular, GA 3β-hydroxylase (Ls3h1) gene expression was induced within 2 h of incubation after red-light treatment, and strict photoreversibility of its regulation was observed. Our results suggest that phytochrome regulates the level of GA 3β-hydroxylase transcripts to increase the amounts of GA_1 in the lettuce seeds.

We observed a decrease in the transcript level for Ls20ox2 within 3 h of incubation after far-red-/red-light treatment. This decline may have been the result of regulation by phytochrome, suppression by Pfr, or negative-feedback regulation by increased GA_1 after far-red-/red-light treatment. With regard to the regulation of elongation growth (described above), it has been suggested that Pfr might suppress GA biosynthesis. GA 20-oxidase transcript levels are subject to feedback regulation (Phillips et al., 1995; Martin et al., 1996; Toyomasu et al., 1997); GA-deficient mutants accumulate a high level of 20-oxidase mRNA, which is markedly reduced by application of bioactive GA. Our preliminary experiments showed that, similar to Ls20ox2, the expression of the Ls20ox1 and Ls3h1 genes, which were not negatively regulated by far-red-/red-light treatment, was markedly decreased by treatment with GA_1 (data not shown). Furthermore, we cannot yet say whether the endogenous GA_1 generated within 3 h of incubation after a far-red-/red-light treatment is sufficient for negative feedback regulation to achieve the observed decrease in the Ls20ox2 transcript levels. Expression of these genes may be developmentally regulated during germination, but they are also potentially subject to feedback regulation by application of bioactive GA. These points can be considered in detail only after determining which tissues these genes are expressed in and where GA_1 is located.

We propose a molecular mechanism by which photoblastic lettuce seed germinates by an upregulation of active GA biosynthesis, which is summarized in Figure 5. Expression of genes encoding GA 20-oxidases, producing GA_{20}, the immediate precursor of GA_1, is induced after an initial 3-h imbibition in the dark, and a red-light treatment converts Pr to Pfr, which upregulates gene expression of GA 3β-hydroxylase. This results in an increase in active GA_1, which induces germination. In spinach (Wu et al., 1996) and Arabidopsis (Xu et al., 1997), both long-day rosette plants, photoperiod regulates the expression of a GA 20-oxidase gene in stem tissues, increasing the levels of bioactive GAs that promote bolting. Photoperiod is thought to be regulated by several factors in response to light conditions (Kendrick and Kronenberg, 1994), and phytochrome is only one of these factors. Because the effect of a light break on the regulation of any GA 20-oxidase gene by photoperiod has not been reported, the role, if any, of phytochrome in this process is unclear. Our study is the first clear demonstration, to our knowledge, that phytochrome can regulate gene expression of GA-biosynthetic enzymes. Further investigations, including protein analysis with antibodies against these enzymes, examination of tissue-specific expression of these genes by in situ hybridization, and promoter analysis of the Ls3h1 gene, will provide more information on the regulation of GA biosynthesis by phytochrome in lettuce seeds.

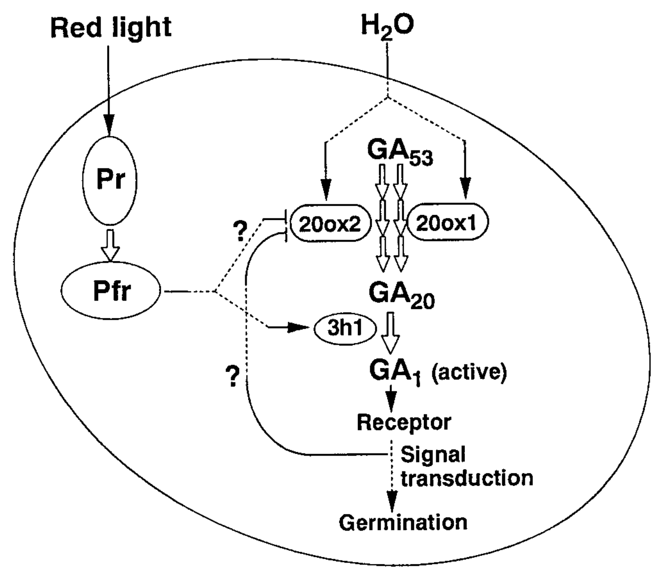

Figure 5. Model of the mechanism of germination of photoblastic lettuce seed. Evidence for this model is described in the text. Open arrows indicate conversion of substances; closed arrows indicate positive action; T-bars indicate negative regulation; question marks indicate unclear relationships; and broken lines indicate more than one process.

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